

The University of Chicago

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CONTROLLING OXIDATION-REDUCTION
POTENTIAL AND ITS APPLICATION
IN THE STUDY OF ANAEROBIOSIS

A PART OF A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

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An Electrolytic Method for Controlling Oxidation-Reduction Potential and Its Application in the Study of Anaerobiosis¹

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INTRODUCTION

The idea that the prevention of growth of obligate anaerobes by air may be determined by a certain level of oxidation-reduction potential, rather than by a specific effect of oxygen, logically followed the work of Clark and others (1) and was formulated by Quastel and Stephenson (2). The disparity between the limiting Eh levels for the growth of a variety of anaerobes as reported by several authors (3) is due, we feel, to the failure to maintain the Eh levels constant for a sufficient length of time. More convincing to us is the work of Knight and Fildes (4) on the limiting Eh level for the germination of spores of *Cl. tetani*, +0.110 volt at pH 7.2; and of Vennesland and Hanke (5) who reported that *Bacteroides vulgatus*, a non-spore-forming obligate anaerobe, does not grow at an Eh level more positive than 0.150 volt at pH 6.6; while at more negative potentials this organism grows regularly on a suitable medium. That the addition of reducing agents promotes the growth of obligate anaerobes (6) and the action of many enzymes (7), has frequently been pointed out. More recently the actions of papain (8), urease (9), and phosphatase (10) have been shown to depend upon certain Eh levels, which differ widely from each other. It appears, then, that oxidation-reduction potential, like hydrogen ion activity, is a determining factor in many enzyme actions, and it is to be expected that Eh, like pH, values and the factors which control them will be found to be of fundamental importance in many biological phenomena and metabolic reactions. Our investigations on anaerobes provide a striking illustration.

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Many observations on the Eh values of biological systems (11) have been reported, and in bacterial systems particularly the effect of growth on oxidation-reduction potential has been studied (12). In our work this point of view has been reversed, in that we are studying the effect of controlled Eh levels on growth. In order to keep the Eh value of an inoculated medium constant, it is necessary to counteract the reducing agents which are liberated by the growing system, and this can be accomplished by the controlled addition of oxidizing agents. Knight and Fildes (4) and Vennesland and Hanke (5) controlled the oxidation-reduction potential of their cultures by means of the proportion of oxygen to nitrogen which was continually passing through the medium.

In this study the potential is controlled by another method, namely direct current electrolysis. By placing in the medium a large platinum electrode which is attached to the positive pole of a direct current, while the negative pole is attached to a similar electrode in another solution which makes a sterile liquid junction to the medium, it is possible to liberate oxidizing agent (or remove reducing agent) at the electrode surface in the medium, and thus make the oxidation-reduction potential of the medium more positive. By attaching the electrode in the medium to the negative pole of the electrolyzing current, the medium may be made more negative. By choosing properly the current and duration as well as the direction of this electrolysis one can achieve a fine control over the oxidation-reduction potential of the solution. A feature of this method is that no extraneous or foreign substances need be added; the electrolysis achieves its control by the oxidation of such reducing agents or the reduction of such oxidizing agents as are inherently in the system. At the same time the gas tension may be chosen at will: pure N_2 , or air, or any mixture of these or other gases.

In this way it is possible to show that certain obligate anaerobes will grow in a continuous current of air, if the oxidation-reduction potential is kept sufficiently negative by direct current electrolysis. On the other hand, under completely anaerobic conditions, these obligate anaerobes will not grow on a nutrient medium if the Eh value is kept above 0.150 volt by positive electrolysis; when subsequently by electrolysis or otherwise the medium is made more negative than Eh 0.150 volt, growth consistently occurs. The limiting potentials for growth are the same whether controlled O_2 tension or controlled electrolysis is used to maintain the potential level. It is also shown here that the limiting potential for growth of *Cl. sporogenes* varies with pH, being a maximum of Eh

0.150 volt at pH 6.6, and somewhat lower, about Eh 0.135, at pH 6.0 and at 7.0. Finally the observations of Katz and Hanke (13) on the O_2 consumption by growing cultures of *Bacteroides vulgatus* have been confirmed and extended.

MATERIALS, APPARATUS, AND GENERAL PROCEDURE

Organisms and Media

The organisms used in this study were two obligate anaerobes of widely different types: *Bacteroides vulgatus*, non-spore-forming, and *Cl. sporogenes*, which is spore-forming. Cultures of the *Bacteroides vulgatus*, strain Marino, were kindly furnished by Dr. A. H. Eggerth of the Long Island College of Medicine. Cultures of the *Cl. sporogenes* were obtained from the Department of Bacteriology of the University of Chicago. Subcultures were made on Rosenow's brain-broth medium. For the potential studies glucose-nutrient broth was used; which consisted of 10 g. glucose, 10 g. sodium chloride, 5 g. Bacto-peptone, and 3 g. meat extract in 1 liter water adjusted to pH 7.0 before autoclaving. After autoclaving, the pH was about 6.7. Most rapid growth was obtained with inocula of 24-hour cultures of *Cl. sporogenes* and 48-hour cultures of *Bacteroides vulgatus*.

Electrode Vessel and Assembly of Apparatus

The electrode culture vessels (Fig. 1) consist of 4-oz. wide-mouth bottles, each of which contains 75 ml. of medium and is fitted with a rubber stopper in which holes are drilled to support the following: A—gas inlet tube, B—inoculating tube and gas outlet, C—two platinum wire electrodes, D—platinum foil electrode, E—burette with capillary tip, rubber connection, and pinch clamp, F—two salt bridges, and G—a short glass tube, 11 mm. outside diameter, which serves as the support for the glass electrode. The salt bridges consist of two L-shaped glass tubes, 5 mm. inside diameter, joined by rubber tubing with space for a screw clamp. Before autoclaving, the distal end of each salt bridge is immersed in a vial containing 3 per cent agar and 1 per cent NaCl, and, without removing air from the bridge, the clamp is closed. During the autoclaving the air is driven out, the bridges fill with fluid, agar on one side and medium on the other, so that after cooling a sterile liquid junction is made simply by opening the clamp. The vials with solid agar are removed and the free ends of the bridges are ready for use. The glass electrode, H, a small bulb sealed on the end of a glass tube 7 mm. outside diameter (see Vennesland and Hanke [5], p. 141, also Hanke [14], pp. 30-33) must be narrow enough to slip through the glass support, G. A short rubber sleeve fits over the glass electrode tube and makes an air-tight joint when inserted into the support, G. The glass electrode is sterilized by immersion for 5 minutes in 0.1 per cent $HgCl_2$, then it is rinsed with sterile distilled water, and

at once immersed in the previously autoclaved vessel containing the culture medium.

All experiments were carried out with the culture vessels inside of a special, electrically shielded incubator, consisting of a copper box, which serves as electrical shield, inside of a transite box electrically regulated at 33°C . By means of a copper tube the shield is made continuous with a vacuum tube potentiometer. We used a Hellige 7030 vacuum tube galvanometer, and a Leeds and Northrup 9655 potentiometer. From each platinum wire or glass electrode in the culture

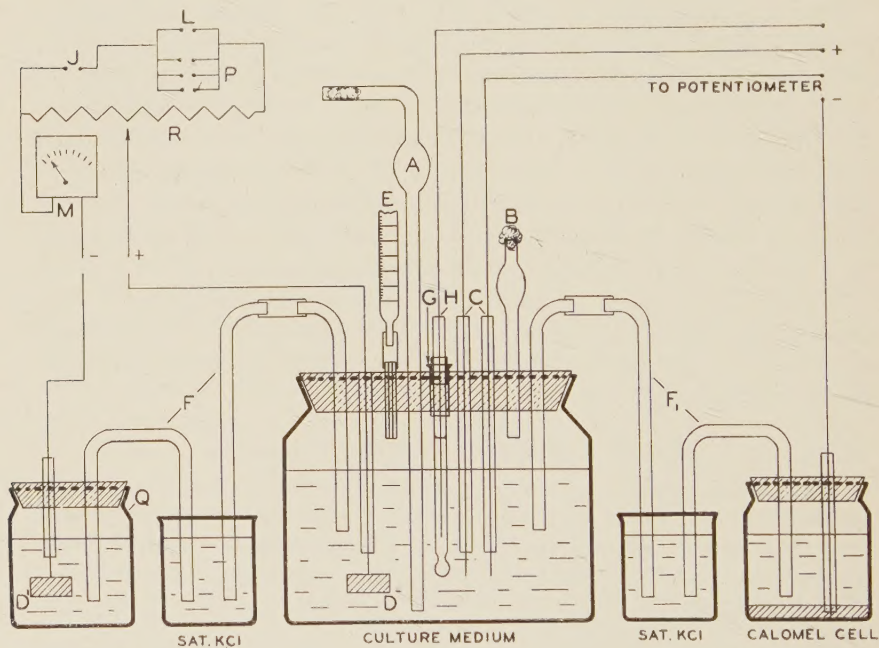


FIG. 1

vessel, by means of spring clips and insulated wires, connection is made to one of a series of permanent binding posts on the inner wall of the copper box, and each of these binding posts may be connected at will through a special selector switch, which can be manipulated from the outside of the box, to the binding posts of the vacuum tube galvanometer. In this way any one of the many electrodes may be instantly chosen for a reading without opening the box, or in any way manipulating the electrode vessels.

The electrolysis circuit, see Fig. 1, is entirely independent of the circuits for reading the potentials. Any one of a number of current sources and combination of resistances may be used. In our electrolysis circuit, the 110 volt direct current J, is led in parallel through a lamp, L, and a 3-heat 8-ampere hot plate, P, and then

in series through a 100-ohm rheostat, R. The current from the rheostat (one fixed and one movable contact) is used for the electrolysis, one pole of which is joined to the large foil electrode, D, in the culture medium, and the other through the milliammeter, M, to the large foil electrode, D', in the bottle, Q, containing saturated KCl. By altering the hot plate switch, and the movable contact of the rheostat, the voltage, and thus the current, in the electrolysis circuit may be varied at will. The maximum electrolysis current, with a single salt bridge of about 5 mm. bore, was about 20 milliamperes, with a voltage of about 30. More recently, with wider, shorter, and multiple bridges, this current has been increased to 120 milliamperes.

From the large foil electrode in the electrode vessel, connection is made through a spring clip to an insulated copper wire which protrudes and is held firmly about 1 cm. outside the transite box. A similar protruding wire makes connection to the large foil electrode in the saturated KCl which in turn is joined electrically through a salt bridge to the electrode vessel. Whenever the medium is to be electrolyzed, the electrolysis wires from the rheostat and the milliammeter ending in the spring clips with labeled polarity are connected to the two protruding wires leading to the large foil electrodes. While taking readings on the potentiometer these electrolyzing leads must be disconnected, because the induced currents disturb the sensitive vacuum tube measuring device.

Use of Dye to Poise the Culture Media

In order to improve the agreement between the readings given by two shiny platinum electrodes in the same culture medium, 2 ml. of 0.03 per cent toluylene blue were added to each vessel (75 ml. of medium). For preparing this solution, about 3 mg. solid dye are put into a dry vial, and after plugging the vial with cotton, all is heated at 110°C. over night. After cooling, 10 ml. sterile water are added, and the mixture is stirred for half a minute to cause solution. Heating the dry dye above 120° or autoclaving in solution causes decomposition. Without dye the readings given by two electrodes usually differ by 10 to 50 millivolts; after adding the dye the electrodes agree within two or three millivolts, when the reading is within 50 millivolts E_0 value of the dye (that is, between E_h of +0.100 to +0.200 volts at pH 6.5). In experiments where growth occurred, the poisoning effect of the dye gradually decreased and after six to ten hours entirely disappeared, due apparently to some chemical destruction of the dye. In such cases the color of the dye did not return even at potential levels where the dye should have been com-

pletely oxidized. Except for the effect of the oxidized form of the dye on the Eh, the presence of the dye was not observed to have any effect on the growth of these anaerobes.

Stirring and the Control of Gas Tension

In order to achieve adequate stirring and also to control the gas tension, gas mixtures were continually bubbled through the culture vessels at a rate of about two bubbles per second. The gas entered through the gas inlet tube, A, Fig. 1, and the constant surging of the gas bubbles effectively stirred the medium. For the anaerobic experiments this gas was purified nitrogen, obtained by passing tank nitrogen through glowing copper gauze in a quartz tube. Since the *Bacteroides vulgatus* required CO₂ for growth, the gases were passed through a saturated solution of sodium bicarbonate immediately before entering the culture vessels. While this CO₂ was not necessary for the *Cl. sporogenes*, it was routinely used in all experiments.

Comparison of Experimental and Control Vessels

Usually three vessels were studied simultaneously: two experimental, and one control. In the experimental vessels, the gas mixtures or electrolytic control of potential were chosen so as to maintain a certain level of potential. In the control vessel, a gas mixture of purified nitrogen and CO₂ was circulated without any electrolysis so as to provide optimum anaerobic conditions, while potential and pH were observed every half hour. In the controls, turbidity, our most definite evidence of growth, appeared in from eight to twelve hours, and a comparison of this time with the time required for turbidity development in the experimental vessels, was a criterion for the adequacy of the conditions in the experimental vessels.

Criteria for Growth

In general, growth was indicated by the successive appearance of three phenomena, increase in reducing power, development of turbidity, and fall in pH (see Vennesland and Hanke [5], p. 153). Increase in reducing power means either a marked increase in negativity of potential, or an increase in the amount of positive electrolysis or oxidizing agent that is needed to keep the potential constant. In the experimental vessels with electrolytic control of potential, the pH changes caused by electrolysis were often more rapid than those which could be

caused by growth, and so pH changes could not very well be used as a criterion for growth. Our most reliable index of growth was, after all, turbidity, and although this was a subjective observation, a comparison of the experimental vessels with the positive control on the one hand, and with a bottle of sterile medium on the other, always led to a very definite and semi-quantitative conclusion.

Tests for Contamination

At the end of each experiment, 0.2-ml. samples of each culture vessel were transferred to sterile tubes of nutrient broth saturated with air, and kept, first at room temperature for 24 hours, and then at 38° for 48 hours to test for aerobic contamination. Any tube showing development of turbidity was considered contaminated, and the results from such vessels where growth occurred were discarded. Contamination occurred in about 10 per cent of the vessels.

General Procedure for an Experiment

The successive steps of an experiment follow: The culture vessel containing 75 ml. of glucose nutrient broth and fitted with the rubber stopper containing the platinum electrodes, salt bridges, burette (but not the glass electrodes) is autoclaved for 20 minutes at 15 pounds pressure. The control vessel, not containing the large foil electrode or the burette, and having only one salt bridge, is simultaneously autoclaved. After cooling, a glass electrode, previously sterilized with 0.1 per cent HgCl_2 and washed with sterile water, is inserted into each vessel. The vessels are placed in the shielded incubator at 38°C. The salt bridges are placed in proper position, and the electrodes are joined to their proper binding posts. The stream of gas is started and the potential of each electrode is read and recorded every 10 minutes. The medium is electrolyzed if necessary to adjust the potentials, and acid or alkali is added to adjust the pH to the previously chosen levels. The brain-broth-culture inoculum, 0.2 ml., is added to each vessel, and after one or two more readings, the dye is added, 2 ml. of 0.03 per cent toluylene blue. In the next 12 to 24 hours, readings are taken on the platinum electrodes in the experimental vessels at least once every 10 minutes; and on all glass electrodes and on the platinum electrodes in the control vessels, once every half hour. Depending upon the relation between the observed and the desired potential, electrolysis is administered so as to keep the potential constantly at the desired level within a range of 10

to 20 millivolts. During the early stages of an experiment, little electrolysis is needed, but while vigorous growth is taking place, as much as 15 milliamperes of positive electrolysis for half the time (5 out of every 10 minutes) may be necessary to counteract the reducing tendency of the growing culture, so that the potential remains constant. Care is taken to insure a continuous flow of gas at a rate of one bubble per second. The first indication of growth is an increase in reducing power, and this is followed within an hour or two by a noticeable turbidity, which becomes marked in 2 to 5 additional hours. The time of the first definite appearance of turbidity is called the time of beginning of growth.

DATA ON LIMITING POTENTIAL STUDIES

Studies on Bacteroides Vulgatus

Table I gives the results of four experiments on the relation of electrolytically maintained oxidation-reduction potential and growth in anaerobic cultures of *Bacteroides vulgatus*. Each experiment is divided into periods of 2 to 4 hours, and the average potentials read every 10 minutes for each of these periods, as well as for the entire experiments are calculated. The first three experiments show that at average potentials of +0.135, 0.138, and 0.144, growth consistently occurred in from 10 to 15 hours; whereas at an average potential of +0.158 (Experiment 4) there was no growth in 20 hours. The control cultures with an Eh of about -0.150 showed growth in $8\frac{1}{2}$ to $10\frac{1}{2}$ hours.

In the last two columns of Tables I and II are given the coulombs of electrolysis (calculated from milliamperes and seconds duration) which must be administered in order to keep the Eh constant, and this is a measure of the reducing power of the culture. It will be noted that whenever growth occurs, indicated as + turbidity, there is an increase in the coulombs of positive electrolysis, usually even in the periods immediately preceding the first noticeable turbidity, indicating an increase in reducing power during growth. This is more strikingly shown in Table II with *Cl. sporogenes*.

Studies on Cl. sporogenes

Table II gives the results of three experiments showing the relation between electrolytically maintained oxidation-reduction potential and growth in anaerobic cultures of *Cl. sporogenes*. It is seen that at an average Eh of 0.136 volt there is growth in 13 hours, while at 0.164

there is no growth in 18 hours. In the third experiment there was no growth at an average Eh of 0.155 in 9 hours, but when later the potential

TABLE I

Effect of Electrolytic Control of Potential on Growth of Bacteroides vulgatus in the Presence of Nitrogen

Average Eh	Average deviation	Time interval	pH	Turbidity	Electrolysis in coulombs
<i>mv.</i>	<i>mv.</i>	<i>hours</i>			
128	15	4	6.93 to 6.39	—	1.5
144	4	4	6.39 to 6.08	—	4.4
130	7	2½	6.08 to 5.87	+	1.6
Total.....135		10½			
145	7	4	6.65 to 6.50	—	—0.65
138	5	4	6.50 to 6.46	—	—1.3
130	6	2½	6.46 to 6.30	+	3.5
Total.....138		10½			
141	10	3	5.87 to 6.70	—	1.9
143	5	3	6.70 to 6.60	—	3.8
146	2	3	6.60 to 6.37	—	4.5
144		3	6.37 to 6.02	+	4.5
146		3	6.02 to 5.68	+	6.5
Total.....144		15			
161	4	4	6.68 to 6.63	—	4.7
158	2	4	6.63 to 6.47	—	2.1
157	2	4	6.47 to 6.43	—	2.6
155	4	4	6.43 to 6.33	—	3.8
158	12	4	6.33 to 6.28	—	3.3
138*	3	4½*	6.28 to 6.22	—	1.3
Total.....158		20			

* Potential lowered to determine viability of culture. Not included in final average potential.

Controls showed turbidity and acid formation 8½ to 10½ hours; final pH 4.8 in 22 to 24 hours; potentials reach Eh —0.150 to —0.175.

was put at 0.128 for 4 hours, growth occurred. Control cultures (without electrolysis) showed growth in from 6½ to 8 hours with an average

Eh of -0.110 . The limiting potential for growth of *Cl. sporogenes* at an initial pH of 6.6 is thus seen to be the same as that of *Bacteroides vulgatus*, namely, about 0.150 volt.

TABLE II

Effect of Electrolytic Control of Potential on Growth of Clostridium sporogenes in the Presence of Nitrogen

Average Eh	Average deviation	Time interval	pH	Turbidity	Electrolysis in coulombs
<i>mv.</i>	<i>mv.</i>	<i>hours</i>			
133	6	3	6.78 to 6.63	—	0.57
138	5	3	6.63 to 6.60	—	0.64
134	4	3	6.60 to 6.23	—	4.4
140	18	4	6.23 to 5.28	+	18.6
Total.....136		13			
161	13	4	6.53 to 6.33	—	-0.3
164	1	4	6.33 to 6.28	—	0.7
166	1	4	6.28 to 6.23	—	0.5
165	1	6	6.23 to 6.05	—	1.1
Total.....164		18			
157	7	3	6.72 to 6.62	—	0.56
156	3	3	6.62 to 6.59	—	0.58
151	4	3	6.59 to 6.22	—	4.8
128*	8	4*	6.22 to 5.30	+	20.4
Total.....155		9			

* Potential lowered to determine viability of culture. Not included in final average potential.

Effect of pH on the Limiting Potential

In the preceding studies the pH of the medium gradually fell as growth and positive electrolysis occurred. In order to study the effect of pH on the limiting potential, alkali was added as needed so that the pH was kept approximately constant, that is within a range of 0.2 pH. The immediate effect of alkali addition, and corresponding rise in pH was to cause a more negative Eh; the addition of acid and fall in pH had the reverse effect; and these effects occurred even in the absence of dye.

The effect of acid or alkali addition on the Eh was about the same as on the glass electrode potential. Electrolysis, however, affected the Eh much more (about 5 to 10 times) than it did the glass electrode potential and so a judicious choice of positive electrolysis and alkali addition allowed a reasonably good control of both pH and Eh.

Three pH levels were studied: 6.6, 6.2, and 7.0, using *Cl. sporogenes*, under anaerobic conditions, with electrolytic control of potential. The data (reported in detail in a Ph.D. dissertation, Katz, 1941 [15]) may be summarized thus: the limiting potentials for growth at pH 6.2 (0.139 volt) and at pH 7.0 (0.132 volt) are definitely more negative than at pH 6.6 (0.148 volt). It seems probable that the limiting potential has a maximum at pH 6.6, but more data are needed to prove this.

GROWTH OF OBLIGATE ANAEROBES IN THE PRESENCE OF AIR

Vennesland and Hanke (5) showed that the anaerobe *Bacteroides vulgatus* would grow in air-nitrogen mixtures, provided the oxidation-reduction potential was kept below +0.150 volt, but growth was never observed when the proportion of air to N₂ exceeded 40%, that is, about 8% O₂. It was of interest to determine whether obligate anaerobes can grow in a continuous current of undiluted air when the potential is maintained by negative electrolysis below the limiting potential for growth. Since negative electrolysis involves alkali formation, the electrode vessels were fitted with burettes containing 0.5 N HCl which was added at intervals whenever the pH rose above 7. The rate of flow of air varied from one bubble per second to one bubble every 4 seconds, and these variations made necessary corresponding variations in the amount of electrolysis necessary to keep the potential constant.

In Experiment 1 of Table III with *Cl. sporogenes* nearly the maximum amount of negative electrolysis (about 10 milliamperes for half of the time) was used, and growth occurred in a continuous current of air at an average Eh +0.082. In Experiment 2 of Table III, where less negative electrolysis was used so that the average potential was 0.124, growth did not occur. Table IV shows similar results with *Bacteroides vulgatus*, where there is growth at an average potential of +0.129 but no growth at +0.143. These experiments show that growth of these anaerobes does occur in a continuous current of air, when the potential is maintained sufficiently negative. At first sight they seem to indicate that the limiting potential for growth in air is slightly more negative than it is anaerobically or at low O₂ tensions. However, because of the

TABLE III

Growth of Clostridium sporogenes in Air with Negative Electrolysis

Average Eh	Average pH	Time interval	Turbidity	Electrolysis in coulombs	HCl m.-eq. per 75 ml.
<i>mv.</i>		<i>hours</i>			
95	6.6	4	—	—66.9	0.5
93	6.2	4	—	—66.0	1.2
57	6.4	4	+	—39.9	
Total..... 82	6.4	12			2.6
129	6.4	4	—	—11.6	0
135	6.9	4	—	—12.0	0
131	7.1	4	—	—6.0	0
140	7.3	4	—	—5.9	0
104	6.2	4	—	—18.3	0.5
Total.....124	6.8	20			0.5

TABLE IV

Growth of Bacteroides vulgatus in Air with Negative Electrolysis

Average Eh	Average pH	Time interval	Turbidity	Electrolysis in coulombs	Acid in m.-eq. per 75 ml.
<i>mv.</i>		<i>hours</i>			
145	6.7	4	—	—56.1	0.20
148	6.8	4	—	—13.5	0.10
144	6.7	4	—	—29.2	0.42
137	6.7	4	—	—67.4	0.46
142	6.6	4	—	—46.4	0.14
Total.....143	6.7	20			
136	6.8	4	—	—61.5	0.76
133	6.9	4	—	—34.9	0.24
117	6.8	4	+	—34.6	0.52
97*	6.7*	4*	+	—72.9	0.60
97*	6.8*	4*	++	—49.9	0.27
Total.....129	6.8	12			

* Values not included in final average potential.

violent opposition of two powerful forces, the oxidizing effect of air and the reducing effect of negative electrolysis, the potential and pH could not be well controlled. The pH fluctuated by as much as 1 unit and the oxidation-reduction potentials by as much as 80 millivolts; and the only conclusion which can be fairly drawn is that the limiting potential in the presence of air is approximately the same as under anaerobic conditions.

STUDIES ON O₂ CONSUMPTION OF OBLIGATE ANAEROBES

Katz and Hanke (13) have reported that when *Bacteroides vulgatus* grows in the presence of O₂, it is consumed with a minimal Q_{O₂} of 11. In these experiments, gas mixtures containing 3 to 9 per cent O₂ were continuously circulated from a closed system through a culture medium containing shiny platinum electrodes for reading oxidation-reduction potentials so that conditions for growth could be accurately known and controlled; and the O₂ content was measured at regular intervals by sampling and analyzing the gas mixture. These observations are here confirmed and extended.

In these experiments no dye was added and the culture was not electrolyzed; so the O₂ consumption cannot be related to any catalytic effect of the dye, or to any reducing action of electrolysis. The conditions for growth or no growth are controlled only by the rate at which the O₂-containing gas mixture is circulated through the culture: a slow rate allows a more negative potential and thus permits growth; a more rapid rate makes the potential more positive and thus prevents growth. The relation of O₂ consumption and growth is made very convincing by the observations that whenever there is growth (determined by low potential) in the presence of O₂, there is also O₂ consumption; whenever there is no growth (determined by high potential) at exactly the same composition of medium, inoculum, and O₂ tension, then there is no O₂ consumption.

Table V summarizes the results of O₂ consumption studies. The first four experiments are at potentials more negative than 0.150 volt where turbidity develops, the pH falls, and O₂ consumption occurs. The last two are at potentials above 0.150 volt and here with the same medium and the same inoculum there is no turbidity, no pH change, and no O₂ consumption. The average O₂ consumption was 0.17 ml. O₂ per hour, by 75 ml. culture medium. At the end of four experiments where growth occurred the medium was centrifuged and the residue

washed with water in weighed centrifuge tubes. The average dry weight of the organisms was 15 mg. The Q_{O_2} is thus 11.

It is of interest to compare the chemical equivalents of O_2 consumed by the growing culture with the electrical equivalents of positive electrolysis which are needed to keep a growing culture at constant potential. Since 22.4 liters of O_2 is 4 equivalents, 0.17 ml. is 30 microequivalents, and this is the average O_2 consumed by the growing culture of *Bacteroides vulgatus* per hour. Now from Table I the positive electrolysis needed to keep the growing culture of *Bacteroides vulgatus* at

TABLE V
O₂ Consumption Related to Growth of Bacteroides vulgatus at Controlled Oxidation-Reduction Potential

Average Eh	pH		Time	Per cent O_2 in gas		ml. Pure O_2 consumed by culture	
	Initial	Final		Initial	Final	In time interval	per hr.
<i>mv.</i>			<i>hours</i>				
117	6.5	4.8	13	6.79	5.63	1.43	0.11
110	5.5	5.3	2.5	3.46	3.15	0.81	0.32
73	5.4	5.1	3	5.26	4.78	0.77	0.25
120	5.6	4.6	13	7.80	6.48	0.77	0.06
222	6.4	6.3	14.5	9.41	9.35	0.04	0.00
168	6.5	6.5	8.5	4.54	4.61	(+0.13)	(+0.01)

The volume of gas in the closed system containing the culture medium varied from 250 ml. at the beginning to 50 ml. at the end. The decrease is due to the samples which were withdrawn for analysis.

constant potential varied from 0.7 to 2.2 coulombs per hour. It is likely that the higher coulomb figure realized in the later stages of growth after the third hour is more nearly comparable with the O_2 consumption value, since the O_2 consumption figures were all in prolonged experiments. Since 96,500 coulombs is one equivalent, 2.2 coulombs is 23 micro-equivalents. The correspondence between 30 micro-equivalents of O_2 and 23 micro-equivalents of positive electrolysis is as close as could be expected, and indicates by each of these two methods of measurement that the reducing power of this anaerobe is the same. From Table II, it is seen that the reducing power of an actively growing culture of *Cl. sporogenes* on the same nutrient broth

medium is about 5 coulombs per hour, and this is more than twice as great as that of *Bacteroides vulgatus*; its O₂ consumption was not measured.

DISCUSSION

The significance of a limiting oxidation-reduction potential for growth of obligate anaerobes and its place in a theory of anaerobiosis has been discussed by Vennesland and Hanke (5). We believe that the growth of these obligate anaerobes depends upon enzymes which have reversibly oxidizable and reducible groups; that these enzymes are active only in the reduced form; and that the reversible oxidation and reduction of these groups occurs for a given pH at a certain level of oxidation-reduction potential. The separation of these enzymes from the bacterial cells, and the demonstration of the relation of their activity to the oxidation-reduction potential of the solution, are problems for the future. Of interest here is that the limiting potential which Sizer and Tytell (9) report for urease, is the same, 0.150 volt at pH 6.8, as that which we found for the growth of these obligate anaerobes. It is also very close to the Eh of 0.160 volt which Lipmann (16) reported caused a marked inhibition of the rate of fermentation of yeast maceration juice. These potentials are, however, much more negative than the limiting potentials reported by Reiss for papain (8), by Sizer (10) for phosphatase, and also more negative than the potential levels which must be involved in the inhibition of the action of carbonic anhydrase (Kiese and Hastings [7]).

Preliminary unpublished experiments show that other obligate anaerobes do not have the same limiting potentials for growth as those found for the two organisms studied here. For *Cl. welchii* and *Cl. histolyticum* we find limiting Eh values of 0.165 and 0.090, respectively, at pH 6.6. The more negative limit for *Cl. histolyticum* may be related to the fact that in our medium *Cl. histolyticum* is an alkali former while the other three, *Cl. welchii*, *Cl. sporogenes*, and *Bacteroides vulgatus*, are acid formers.

The fact that the limiting potential for growth remains the same, while the O₂ tensions which the organisms will tolerate vary widely, indicates that the limiting factor is Eh and not O₂ tension. For instance, immediately after inoculation of a medium with an obligate anaerobe, as little as 0.1 per cent O₂ is enough to prevent all growth. But after growth is well started in the absence of O₂, the organisms will tolerate

and continue to grow in an atmosphere of 5 to 8 per cent O_2 . And with adequate negative electrolysis the organism will grow in a current of undiluted air. The constant factor distinguishing growth from no growth at all these O_2 tensions is the Eh level: immediately after inoculation when the reducing power of the culture is very little, 0.1 per cent O_2 is enough to keep the Eh more positive than 0.150 volt and so there is no growth; after growth is well started, 5 per cent O_2 is not enough to counteract the vigorous reducing power of the growing organisms, not enough to keep the potential more positive than 0.150 volt, and therefore growth continues. We conclude that O_2 *per se* has little if anything to do with the prevention of growth of obligate anaerobes; it influences their growth only to the extent that it affects the oxidation-reduction potential. The fact that the same limiting potential for growth is found in the presence of a reversible oxidation-reduction indicator (toluylene blue) as is found without such dye adds confidence to the validity of the figure.

The observations on O_2 consumption are, we believe, the first to show that obligate anaerobes consume O_2 during growth. This is particularly interesting in view of the discussion by Broh-Kahn and Mirsky (17) to the effect that there is no evidence that obligate anaerobes ever consume oxygen, and that strict anaerobes contain no catalysts for the activation of oxygen. Warburg and Christian (18) have shown that certain anaerobes have in fact the highest concentration of yellow enzyme found in any cells. If the oxygen consumption observed in the anaerobes in our studies is catalyzed by the yellow enzyme, it would involve production of hydrogen peroxide. Venesland and Hanke (5) were unable to demonstrate hydrogen peroxide in cultures of *Bacteroides vulgatus*, exposed to air. On the other hand, Sherman (19) showed that some anaerobes contain catalase, so the absence of peroxide would follow if catalase is present in the *Bacteroides* culture.

The Q_{O_2} of 11 is a minimal value. This is very close to the Q_{O_2} of 6 to 12 calculated by Stickland (20) from observations on methylene blue reduction in a Thunberg tube by *Cl. sporogenes*. It is of the same order of magnitude as the values (3 to 20) reported by Krebs (21) for resting mammalian tissue. For aerobic micro-organisms, values of from 13 to 150 are reported (22). When we recognize that our value of 11 is a minimal value based on the final weight of organisms, and that the actual Q_{O_2} based on the average weight of organisms would probably

be about twice this value, we see that the Q_{O_2} of anaerobes is of the same order of magnitude as that of aerobes.

The electrolytic method of controlling oxidation-reduction potential is recommended whenever it is desired to maintain a constant level of potential in the face of the variable addition or removal of oxidizing or reducing agents; or whenever carefully graded quantities of oxidizing or reducing agent are to be administered *without the addition of a foreign substance*; that is, by the mere oxidation of such reducing agents, or the reduction of such oxidizing agents, as are already present in the solution. It should also be useful in electrometric titrations, where, of course, the added oxidizing or reducing agent is not a new substance from a burette, but simply a measured number of coulombs of electric current. It should be of especial value in biological investigations.

SUMMARY

1. An electrolytic method for controlling the oxidation-reduction potential of aqueous solutions is described, by which it is possible to maintain constant the Eh of bacterial cultures without the addition of any foreign agents to the medium.

2. From studies at a variety of potentials and O_2 tensions of cultures of *Bacteroides vulgatus* and *Cl. sporogenes*, it is found that the limiting Eh for the initiation of growth of these obligate anaerobes is 0.150 volt at pH 6.6. At pH 7.0 and at pH 6.2 the limiting Eh is more negative by about 15 millivolts.

3. When the potential is kept sufficiently negative electrolytically, these anaerobes will grow in a continuous current of air.

4. Since these limiting potentials are independent of marked variations in O_2 tension, we feel that the limiting factor in the growth of these anaerobes is the Eh level and not O_2 tension.

5. Studies on O_2 consumption at controlled oxidation-reduction potential have shown that when *Bacteroides vulgatus* grows in the presence of O_2 , O_2 is consumed with a minimum Q_{O_2} of 11.

6. The reducing power of an actively growing culture of *Bacteroides vulgatus*, about 30 micro-equivalents per hour (per 15 mg. dry weight of organisms in 75 ml. of culture medium), is approximately the same, whether measured by O_2 consumption or by the coulombs of positive electrolysis needed to keep the Eh constant.

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